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Research Article

Determination of Tetracycline and Fluoroquinolone Antibiotics at Trace Levels in Sludge and Soil

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This work describes the development of a sensitive analytical method to determine simultaneously traces of tetracycline and fluoroquinolone antibiotics in sludge and soil, based on PLE extraction, followed by SPE purification and finally an analysis by LC-MS/MS. Recoveries were greater than 87% in the case of fluoroquinolones and between 25.4 and 41.7% for tetracyclines. Low relative standard deviations (<15%) were obtained in both matrices. The limits of quantification were comprised between 1.1 and 4.6 ng/g and between 5 and 20 ng/g in soil and sludge, respectively. The method was then successfully applied to the analysis of the target antibiotics in sludge as well as soil that received spreading. The substances most frequently found and with the highest levels were fluoroquinolones with concentrations exceeding 1,000 ng/g in several samples of sludge and up to 16 ng/g in soil.

1. Introduction

The presence of many organic substances in all compartments of the environment, from our domestic life as well as from agricultural and industrial activities, is now a proven fact [1–4]. Among the compounds that represent a source of concern to the scientific community are veterinary and human antibiotics. Many prescriptions are given to treat bacterial infections in human or animals and antibiotics are also used for growth promotion [5–7]. After their administration, antibiotics are partially metabolized by the body [5, 8] and subsequently excreted and pass through treatment plants where they can appear recalcitrant to treatments [9–11]. These compounds are then present in the effluent or sewage sludge and also in the manure applied to agricultural land. The dissemination of antibiotics into the environment can have adverse effects over time. Indeed, they can induce pathogen resistance [12–14] and be harmful to ecosystems and human health.

According to a report from ANMV (French National Agency of Veterinary Medicine), the total sales volume in France amounted to 782 tons of veterinary antibiotics in 2012 [15]. This is the lowest tonnage recorded since tracking began. However, given the differences of activity of antibiotics and their varied doses, tonnages sold do not accurately reflect the use of antibiotics. Indeed, recent antibiotics are generally more active and require the administration of a lower amount. In human medicine, the quantitative consumption level has increased continuously since 2010, according to ANSM (French National Agency for Medicines Safety and Health Products). Thus, it reaches 32.3 numbers of defined daily doses per 1,000 inhabitants per day.

Two families of antibiotics are especially noteworthy: tetracyclines and fluoroquinolones. Tetracyclines represent almost half the sales of antibiotics for veterinary purpose in France [15]. These molecules have the characteristic of having four linearly annelated six-membered rings with a characteristic arrangement of double bonds (Table 1). They are substituted with more or less hydrophilic groups (hydroxyl, amide, or ketone) which form chelates with the cations (Ca^{2+}, Mg^{2+}) naturally present in the environment [16, 17]. Consequently tetracyclines will tend to complex with di- or trivalent cations present in the soil and thus to be strongly adsorbed on this matrix and therefore not leached. In addition, these molecules have an amphoteric character. Indeed, they have three ionizable sites and can exist in cationic, anionic, or zwitterionic forms. The adsorption

Table 1: Physicochemical properties of selected substances.

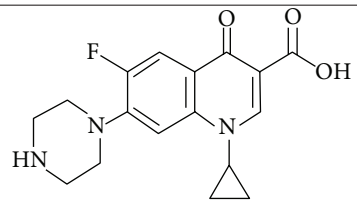
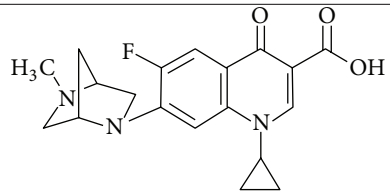
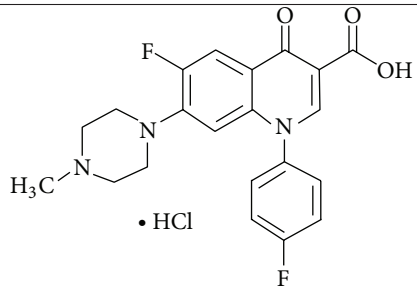
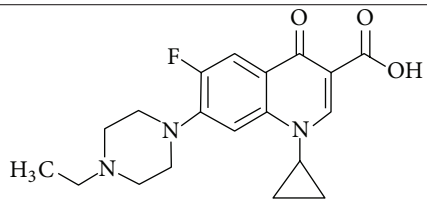
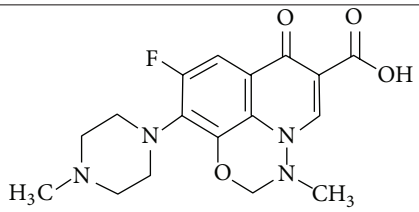
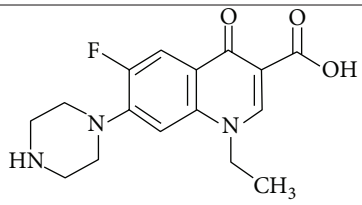
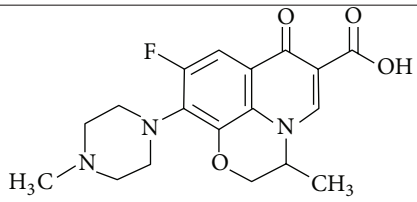
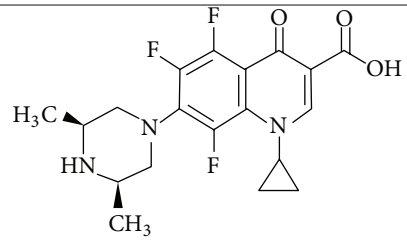
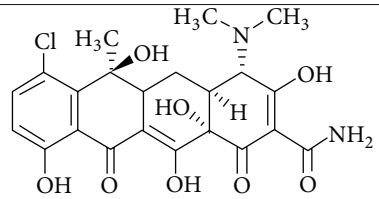
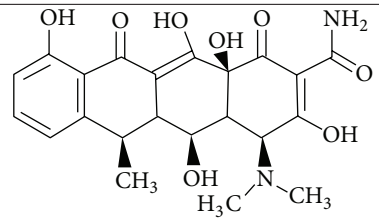
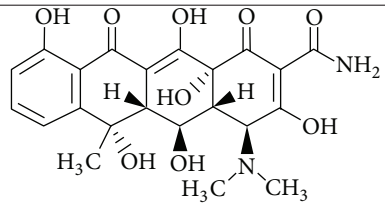
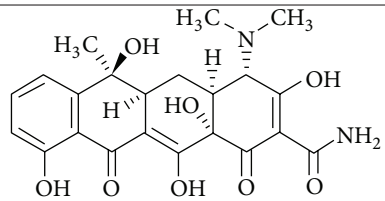
Compound	Formula	Log K_{ow}	Water solubility (mg/L)	pK_a
Ciprofloxacine		0.28	30000 (20°C)	pK_{a1} = 5.90 pK_{a2} = 8.89
Danofloxacin		−0.3	172000 (25°C)	pK_{a1} = 6.07 pK_{a2} = 8.56
Difloxacin		0.89	1330 (25°C)	pK_{a1} = 4.33 pK_{a2} = 9.05
Enrofloxacin		0.70	3400 (25°C)	pK_{a1} = 6.27 pK_{a2} = 8.30
Marbofloxacin		−0.83	7520 (25°C)	pK_{a1} = 5.77 pK_{a2} = 8.22
Norfloxacin		−1.03	178000 (25°C)	pK_{a1} = 6.23 pK_{a2} = 8.55
Ofloxacin		−0.39	28300 (25°C)	pK_{a1} = 5.97 pK_{a2} = 8.28

Table 1: Continued.

Compound	Formula	Log K_{ow}	Water solubility (mg/L)	pK_a
Orbifloxacin		−0.78	909 (25°C)	pK_{a1} = 5.95 pK_{a2} = 9.01
Chlortetracycline		−0.62	630 (25°C)	pK_{a1} = 3.30 pK_{a2} = 7.44 pK_{a3} = 9.27
Doxycycline		−0.02	630 (25°C)	pK_{a1} = 3.50 pK_{a2} = 7.70 pK_{a3} = 9.50
Oxytetracycline		−0.90	313 (25°C)	pK_{a1} = 3.27 pK_{a2} = 7.32 pK_{a3} = 9.11
Tetracycline		−1.30	231 (25°C)	pK_{a1} = 3.30 pK_{a2} = 7.68 pK_{a3} = 9.30

of tetracyclines on the soil strongly depends on the pH; the cation exchange interaction is also known to be a very important adsorption process [18]. These substances present low log K_{ow} (octanol-water partition coefficient) values as shown in Table 1. Log K_{ow} is currently used to characterize or evaluate the presence of organic micro pollutants in the various phases of the environment. It is a measure of the equilibrium concentration of a compound between octanol and water that indicates the potential for partitioning into soil organic matter.

The volume of sales of fluoroquinolones for veterinary use is lower than tetracyclines (0.6% of total sales). However, total sales of fluoroquinolones tend to increase gradually. It went from 3.3 to 4.9 tonnes between 1999 and 2012 [15]. Of the 14 years of monitoring, the level of exposure of animals to fluoroquinolones has increased by a factor of 2 [15]. Moreover, they are often used in human. Fluoroquinolones have been categorized as being of the highest priority for risk management among antimicrobials in a report of the WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance [19]. This document emphasizes that fluoroquinolones are the sole or one of the few alternatives to treat serious disease such as severe *Salmonella* and *E. coli* infections. But these antibiotics are known to select for fluoroquinolone-resistant *Salmonella* and *E. coli* in animals.

Quinolones have a bicycle structure with nitrogen at position 1, a carboxylic acid function in position 3, and a carbonyl in position 4 (Table 1). Fluoroquinolones contain, in addition, fluorine in position 7. The presence of fluorine as well as a carboxylic acid functional group imparts a polar character (log K_{ow} between −1.03 and 0.89). As tetracyclines, these substances tend to form stable complexes with di- and trivalent cations (Ca^{2+}, Mg^{2+}, and Al^{3+}). Interactions are also being established between the ion, the oxygen of the ketone in position 4, and the carboxylic acid function (position 3) [20]. Fluoroquinolones will therefore tend to adsorb on

solid matrices. In addition, these compounds have mostly two ionizable functions, the carboxyl group and the nitrogen atom in the ring. Different shapes, cationic, anionic, and zwitterionic, will be present in the medium according to the pH, which will affect their mobility or transfer in soil.

Several methods have already been published to analyze fluoroquinolone and/or tetracycline antibiotics in soil or sludge. Assuredly, the most difficult and time-consuming task for the determination of these substances in solid environmental matrices is the sample preparation, which often combines one or more extraction and purification procedures. Pressurised liquid extraction (PLE) [21–23], ultrasonic-assisted extraction [22, 24, 25], or techniques based on the use of microwaves [22, 24, 26] have been described. An additional purification step is necessary to eliminate residual interfering substances that affect the quality and robustness of the analysis. Solid phase extraction (SPE) appears as the most suitable technic [21, 23–25] since it is fast and efficient and the range of phase available is very wide. Moreover, it requires a low quantity of solvent and presents limited risk of sample contamination.

Few procedures have been dedicated to the quantification of both tetracyclines and fluoroquinolones and, above all, there is lack of method allowing the evaluation of the transfer of these compounds from sludge to soil. Therefore, detection and quantification with few steps and in a reasonable time of these antibiotics are a necessity to evaluate their occurrence and levels in sludge and soil or to estimate the potential of transfer of these compounds from sludge through the environment.

In this paper, we describe the development of a sensitive method capable of determining simultaneously traces of tetracycline and fluoroquinolone antibiotics in sludge and soil. This method is based on PLE extraction, followed by SPE purification and finally an analysis by LC-MS/MS. The method is then successfully applied to the analysis of the target antibiotics in both sludge and soil that received spreading. Our approach was a bit different from what is usual since we have developed the method of extraction from 13 substances to better understand the role of each of the optimization settings. However, once optimal conditions are identified, we have validated and applied the method on real samples only for five substances most likely to be found in the environment.

2. Experimental

2.1. Chemicals and Materials. High purity (>98%) analytical standards were used. Tetracycline, oxytetracycline, chlortetracycline, doxycycline, minocycline, norfloxacin, danofloxacin, enrofloxacin, ofloxacin, ciprofloxacin, marbofloxacin, orbifloxacin, and difloxacin were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France).

Stock solutions of each standard were prepared at concentrations of 200 mg/L in methanol and were stored at −23°C in amber glass. Working solutions were prepared by the appropriate mixture and dilution of the stock solutions.

Methanol (MeOH), acetonitrile (ACN) (LC-MS grade), dimethylsulfoxide (DMSO), and acetone (HPLC grade) were purchased from Sigma-Aldrich. Pure water was obtained from a MilliQ device from Millipore (Saint-Quentin-en-Yvelines, France). Formic acid (98%, LC-MS grade) and citric acid monohydrate were purchased from Sigma-Aldrich while sodium hydroxide was obtained from Prolabo (Paris, France).

2.2. Solid Samples. For the development and validation of the method, clay-loam soil that was never treated with manure or sludge was used. The soil sample was passed through 3 mm sieve to remove coarse particles. It was subsequently ground in a mortar and passed through 0.63 mm sieve to obtain a homogeneous sample. For the application to real samples, soils were collected in amber glass bottles, at a depth of 10 cm, and then were stored at −23°C before the pretreatment and analysis.

For the development and validation of the method in sludge, digested sludge was used. It was frozen in a previously burnt aluminium tray and then freeze-dried for 48 h. It was finally ground in a mortar and sieved to 0.25 mm. For the application to real samples, sludge measurements were carried out in glass bottles by authorized personnel of each treatment plant, according to the norm NF EN ISO 5667-15 (Oct. 2009) dealing with the conservation and treatment of sludge.

2.3. Extraction and Clean-Up. PLE experiments were performed with the system ASE 350 from Thermo Scientific (Villebon-sur-Yvette, France). 11-mL PLE cell was filled with 5 g of soil or 0.5 g of sludge and completed with diatomaceous earth from Sartorius (Germany).

The extraction procedures were performed at the temperature of 80°C, the pressure of 120 bar, 10 min of static time, and 2 cycles. The composition of the solvent was MeOH, ACN, and 0.2 M citric acid (pH = 4.5) in the proportions 40/40/20. At the end of the two cycles, the extract was supplemented to 500 mL with MilliQ water.

After PLE, the clean-up was performed by SPE using the Thermo Scientific Dionex AutoTrace 280 Instrument with StrataX cartridges (200 mg/3 mL) from Phenomenex (Le Pecq, France). The cartridge was previously conditioned with 5 mL MeOH and then 5 mL H_2O. The volume of 500 mL of reconstituted PLE extract was loaded in the cartridge at the flow rate of 5 mL/min. The cartridge was subsequently washed with 5 mL H_2O and then dried for 15 min under a stream of nitrogen and finally eluted with 6 mL MeOH at the flow rate of 2 mL/min. Then, 100 μL of DMSO was added to the extract before the evaporation of MeOH under a gentle stream of nitrogen at the temperature of 40°C. Finally, 375 μL H_2O and 25 μL ACN were added prior to LC-MS/MS analysis.

2.4. Analysis by Liquid Chromatography-Tandem Mass Spectrometry. The liquid chromatographic separation was performed on an Agilent Series 1100 HPLC system from Agilent Technologies (Les Ulis, France) equipped with a binary pump, a degasser, and a column oven. The separation was performed with a Poroshell 120 EC-C18 (100 × 2.1 mm, 2.7 μm) column from Agilent Technologies preceded by column prefilter KrudKatcher from Phenomenex. The mobile phase was composed of 0.1% formic acid in MilliQ water (A) and 0.1% formic acid in MeOH (B) with the following

Table 2: Precursor and product ions, collision energy (CE), and declustering potential (DP).

Compounds	Precursor ion → product ion (CE (eV))	DP (V)
Tetracycline	445 → 410 (25) and 445 → 154 (35)	36
Oxytetracycline	461 → 426 (25) and 461 → 443 (19)	36
Chlortetracycline	479 → 444 (27) and 479 → 462 (23)	36
Minocycline	479 → 441 (27) and 479 → 352 (23)	36
Doxycycline	445 → 428 (23) and 445 → 154 (41)	41
Ciprofloxacin	332 → 288 (23) and 332 → 231 (49)	51
Enrofloxacin	360 → 316 (25) and 360 → 245 (35)	51
Ofloxacin	362 → 261 (35) and 362 → 318 (23)	51
Norfloxacin	320 → 276 (27) and 320 → 231 (51)	61
Danofloxacin	358 → 340 (27) and 358 → 82 (67)	26
Marbofloxacin	363 → 72 (37) and 363 → 320 (23)	21
Orbifloxacin	396 → 295 (33) and 396 → 352 (23)	31
Difloxacin	400 → 356 (25) and 400 → 299 (35)	41

gradient: 85% (A) for 4 min and from 85% to 0% (A) in 2 min. At the end of the analysis, the column was rinsed by 100% (B) for 5 min. The flow rate was 0.4 mL/min, the oven temperature was 60°C, and the injection volume was 20 μL.

The LC system was coupled to a triple-stage 3200 QTRAP (ABSciex, Les Ulis, France) with an electrospray ion source used in positive mode. The MS/MS settings and the parameters of the source were optimized by manual infusion and by the flow injection of each standard at 10 mg/L. The source parameters were as follows: 5500 V source voltage, 600°C source temperature, nebulisation gas at 45 psi, and desolvation gas at 55 psi (nitrogen). The MS/MS conditions are summarised in Table 2. The analytes were identified both by their chromatographic characteristics and by their specific multiple reaction monitoring (MRM) fragmentation patterns. Data processing was performed with Analyst software (version 1.5.1).

3. Results and Discussion

3.1. Development of the PLE. During the development of the method based on PLE, several parameters had to be optimized such as the temperature, the pressure, the static time, and the number of cycles, as well as the nature of the extraction solvent. The solvent is the parameter that most affects the extraction efficiency. Therefore, the temperature, the pressure, the static time, and the number of cycles were chosen based on literature data and we optimized the extraction solvent.

Some previous studies have been dedicated to the extraction of tetracyclines and fluoroquinolones from soil by PLE. Relatively low temperatures, comprised between room temperature and 100°C, were used to extract tetracyclines, substances sensitive to thermal degradation [27–29]. In addition, the use of an elevated temperature can lead to another limitation: the coextraction of compounds of the matrix that may interfere with the target analytes [29]. Regarding fluoroquinolones, a temperature of 100°C was generally used [8, 30]. We therefore set a temperature of 80°C. A pressure between 100 and 150 bar is often used for these two families of antibiotics [8, 27, 28, 30]. We therefore set the pressure at 120 bar. A 10-minute static time was generally reported [8, 27, 28, 30]; therefore we chose this time. Finally, 2 or 3 cycles were often mentioned in the literature to extract tetracyclines or fluoroquinolones. We chose to run 2 cycles [27, 28].

With these conditions, we attributed great importance to the choice of solvent to optimize the extraction of both tetracyclines and fluoroquinolones. These tests were carried out from 1 g of soil spiked at 500 ng/g with each substance. The sample was then left for 8 hours at room temperature before the extraction, to allow the solvent to evaporate.

The extracts obtained were relatively loaded with organic matter making their direct injection into the LC-MS/MS system difficult. Therefore, purification by SPE was performed using the polymeric StrataX cartridges. In order to evaluate only the recoveries from the PLE during the optimization, we compared the responses of the solids spiked before the extraction and after the PLE extraction but before the purification step.

As preliminary tests, we evaluated the effectiveness of acetone, MeOH, or mixtures acetone/MeOH (50/50), MeOH/H_2O (80/20), and ACN/H_2O (70/30). The extracts obtained were evaporated and dissolved in 1 mL of 95/5 H_2O/MeOH and then injected into LC-MS/MS. The results showed that only mixtures of organic solvent with water allowed the extraction of a minimum of fluoroquinolones.

We therefore continued the optimization with mixtures of more complex solvents, including an aqueous portion. We first chose to evaluate solvents based on citric acid. Indeed, it was demonstrated that fluoroquinolones and tetracyclines easily form strong complexes with di- and trivalent cations contained between the layers of clay or with the hydroxyl groups at the solid particle surface [8, 27]. In addition, cation exchange, hydrophobic reactions, or hydrogen bond formation could also take place [27], making the extraction of these antibiotics difficult. To reduce these interactions, a complexing agent could be added in the extraction solvent. Citric acid could represent a good choice. Moreover, acidic pH could favor electrostatic repulsion between target substances and soil surface, both protonated.

Preliminary experiments dealing with the choice of the solvent described above put in evidence the notion that fluoroquinolones were better extracted with ACN/H_2O (70/30). On the other hand, tetracyclines appeared to be more extracted from the solid matrix with MeOH/H_2O (80/20). We therefore assessed various compositions of solvents containing acetonitrile, methanol, and citric acid. Jacobsen et al. investigated the amount of citric acid to the tetracyclines extraction from soil [27]; they demonstrated that a concentration of 0.2 M in the aqueous portion of the solvent mixture allowed reaching the most interesting recoveries. We therefore chose to introduce 0.2 M citric acid in the aqueous fraction of each of the mixtures and the equivalent of 0.1 M to 50/50 methanol/acetonitrile. Sodium hydroxide was added to adjust the pH values of 3 or 4.5. The recoveries obtained with the various compositions of solvent (MeOH/ACN/0.2 M citric acid) and pH values are presented in Figures 1(a) and 1(b) for tetracyclines and fluoroquinolones, respectively.

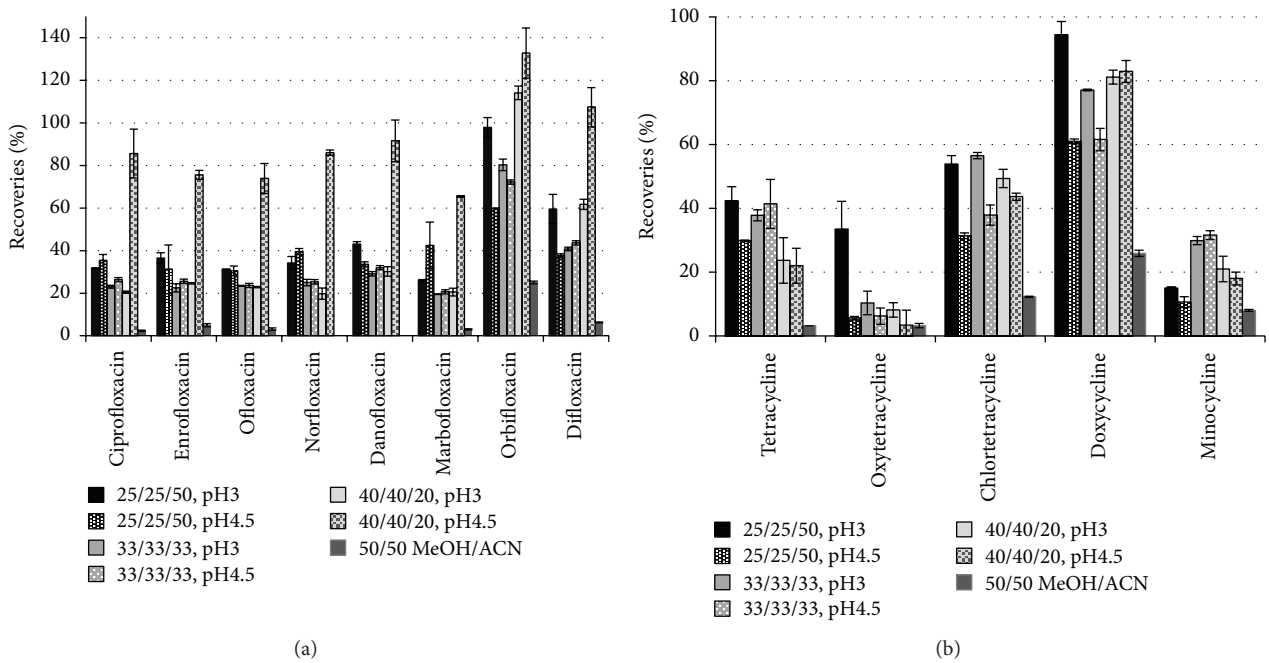


Figure 1: Recoveries obtained for (a) fluoroquinolones and (b) tetracyclines, with various compositions of PLE solvent (MeOH/ACN/0.2 M citric acid) and pH values.

These figures show that the 50/50 mixture of MeOH/ACN containing citric acid very little extracted the target substances. A minimum volume of water was required for extraction of tetracyclines and fluoroquinolones. Moreover, we note that fluoroquinolones were better extracted with the composition 40/40/20 MeOH/ACN/citric acid adjusted to pH = 4.5 (recoveries between 65 and 133%). On the other hand, the extraction of tetracyclines appeared to be less affected by the composition of the solvent and the pH, except in the case of oxytetracycline for which the extraction was favored with the 25/25/50 MeOH/ACN/citric acid composition and the pH = 3. Under these conditions, the choice of the extraction solvent for both families was 40/40/20 MeOH/ACN/citric acid adjusted to pH = 4.5.

3.2. Optimization of the Injection Conditions. In a previous study dealing with the development of a method dedicated to the analyses of pharmaceutical compounds and hormones in sludge by LC-ToF-MS [31], we put in evidence the positive role of DMSO in the response of the target compounds. We showed that DMSO was playing a double role. Firstly, it prevented the complete evaporation of the extract, thereby facilitating the dissolution of the residue in the injection solvent. And secondly, it allowed a significant increase of the ionization of certain substances in the electrospray source in the presence of acidified water.

Therefore, we evaluated the influence of the presence of DMSO on the signal of tetracyclines and fluoroquinolones. Two sets of experiments were conducted: the first one to assess whether DMSO played a role and what was the best proportion in the mixture of solvents and the second one to evaluate the optimal final volume of solvents. For these optimizations, soil was previously spiked at 80 ng/g with each of the target substances and was submitted to the PLE extraction and then the SPE purification as described above. Various volumes of DMSO were added to the extract and MeOH was evaporated under nitrogen. A volume of 50 μL ACN was added and the solvent was made up with water to 1 mL. The response obtained by LC-MS/MS for these various proportions of DMSO is presented in Figure 2. The results indicate that DMSO played a positive role in the response obtained in the case of fluoroquinolones but had no

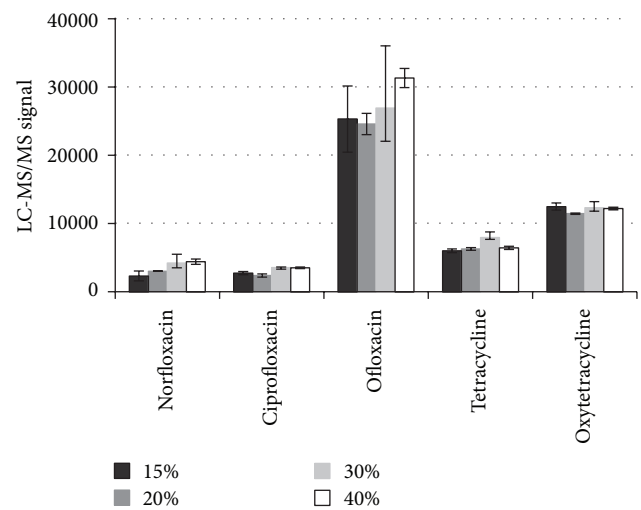


Figure 2: Signal response of some tetracyclines and fluoroquinolones as a function of the percentage of DMSO in 1 mL of extract.

Table 3: Validation parameters: linearity, limits of detection and quantification (MDL and MQL), recoveries (R), and precision (relative standard deviation, RSD) in soil and sludge.

Compounds	Linearity (r^2)		MDL (ng/g)		MQL (ng/g)		R (%)		Intraday precision RSD (%)		Interday precision RSD (%)	
	Soil 1–200 ng/g	Sludge 5–2500 ng/g	Soil	Sludge	Soil	Sludge	Soil	Sludge	Soil	Sludge	Soil	Sludge
Norfloxacin	0.9956	0.9906	0.7	3.8	1.1	12.7	96.6	91.4	1.2	15.0		16.5
Ciprofloxacin	0.9869	0.9911	1.4	6	4.6	20.0	92.6	87.1	1.2	13.1		8.0
Ofloxacin	0.9947	0.9941	0.4	1.5	1.3	5.0	96.9	89.8	7.0	11.0		2.8
Tetracycline	0.9948	0.9917	0.3	2.4	1.9	6.2	38.9	41.7	14.7	1.9		6.7
Oxytetracycline	0.9970	0.9968	0.7	6	2.3	20.0	25.4	33.5	4.7	5.4		15.1

significant effect on the tetracyclines. The best response was generally obtained for a volume of 300 μL. However, the measure was less reproducible with this content than with 200 μL DMSO. Therefore, the following proportions were selected: 20% DMSO, 5% ACN, and 75% H_2O. Second, several total volumes of solvent from 400 μL to 1000 μL were tested with these proportions of DMSO, ACN, and H_2O. The best signal intensities were obtained for a volume of 500 μL. To the best of our knowledge, the influence of DMSO on the MS response of tetracyclines or fluoroquinolones was not evidenced.

Finally, the injection volume was also optimized. The volume could be increased up to 20 μL while maintaining good sharpness of the peaks.

3.3. Performance of the Methods. The validation procedure of the method was carried out using spiked soil or sludge. As it was not possible to find sludge that was free of the target analytes, the sludge samples were previously analyzed and the LC-MS/MS signals corresponding to the target compounds were subtracted. With regard to the soil, analysis of unspiked soil showed that it was free of targeted substances.

The methods were evaluated in terms of linearity, recoveries, and intra- and interday repeatability as well as the limits of detection and quantification.

The linearity was evaluated by assessing the signal response of each compound from spiked samples over a concentration range from 1 to 200 ng/g in soil and from 5 to 2500 ng/g in sludge. The model was evaluated in triplicate on three days. Table 3 indicates that the methods were linear over the specified concentration ranges, in both matrices. The determination coefficients (R^2) were higher than 0.99, except in the case of ciprofloxacin in soil (0.9869).

To evaluate recoveries, the signal obtained for solid samples spiked before the sample preparation ($S_{\text{spiked before}}$) was compared with the signal obtained for solid extract spiked after the sample preparation with MeOH solution containing the target substances at the same concentration ($S_{\text{spiked after}}$), where S_{blank} corresponds to the signal of a nonspiked extract of the sample, according to

$$\text{recovery}\,(\%) = \left(\frac{S_{\text{spiked before}} - S_{\text{blank}}}{S_{\text{spiked after}}}\right) \times 100. \quad (1)$$

Recoveries were different for tetracyclines and fluoroquinolones (Table 3). Indeed, they were greater than 87% in the case of fluoroquinolones but only between 25.4 and 41.7% for tetracyclines. These lower values confirmed the strong interactions that exist between tetracyclines and environmental solid matrices. The difficult extraction of tetracyclines was previously reported by Carvalho et al. [24] that compared the efficiency of vortex agitation, ultrasonic extraction, and microwave assisted extraction but did not obtain recoveries higher than 59% and 42% for tetracycline and oxytetracycline, respectively, in sediment. Similarly, previous methods involving SPE for the extraction did not exceed 27% for the recovery of tetracycline from sludge [21] or 54% for tetracycline and oxytetracycline [23]. Recently, Huang et al. [25] developed a method that allowed recoveries superior to 81% for tetracycline. However, this efficient method was quite complex and time and solvent consuming since it involved extraction overnight by a solvent, then three successive ultrasonic-assisted extraction procedures aided by mechanical shaking, and finally clean-up by solid phase extraction.

Precision was appraised through intra- and interday analyses. It was performed at 80 ng/g and 250 ng/g, in soil and sludge, respectively. It consisted in three replicates conducted on the same day or three different days, respectively, and was expressed as the relative standard deviation (RSD, %). Good intraday precision results were obtained, with RSD values inferior to 15% for all the compounds in both matrices (Table 3).

The limits of detection and quantification of the method (MDL and MQL, resp.) were finally determined. They were defined as the analyte concentration that produced a peak signal of three and ten times the background noise, respectively. The method appeared to be sensitive since MQL values were all inferior to 5 ng/g in soil and inferior to 20 ng/g in sludge (Table 3). These limits were broadly the same order of magnitude as the methods proposed in the literature for the quantification of fluoroquinolones [22, 25, 32]. Only Lillenberg et al. [21] developed a method for their analysis to levels below 2 ng/g in sludge. On the other hand, the limit of quantification of tetracyclines did not fall below 80 ng/g. For the analysis of tetracyclines, the MQL of the developed method are generally lower than or equivalent to those reported in the literature [21–25, 33].

For quantification in sludge or soil, one of the drawbacks is the presence of residual interfering compounds in the

Table 4: Levels (ng/g dry matter) of target tetracyclines and fluoroquinolones measured in sludge and soil that received the corresponding sludge.

Sludge	Sludge 1	Sludge 2	Sludge 3	Sludge 4	Sludge 5	Sludge 6	Sludge 7	Sludge 8	Sludge 9	Sludge 10	Sludge 11	Sludge 12
Norfloxacin	nd	2,055 (157)	nd	937 (41)	132 (8)	74 (3)	nd	130 (5)	84 (7)	1,004 (42)	362 (17)	562 (21)
Ciprofloxacin	nd	3,148 (94)	63 ± (2)	2,786 (115)	251 (11)	55 (2)	nd	356 (15)	123 (5)	379 (16)	576 (28)	755 (33)
Ofloxacin	nd	8,492 (212)	66 ± (3)	2,577 (93)	398 (14)	93 (30)	208 (67)	824 (29)	156 (54)	1,189 (42)	641 (25)	1,152 (39)
Tetracycline	nd	124 (3)	nd	nd	nd	nd	nd	nd	nd	61 (2)	nd	135 (2)
Oxytetracycline	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Soil	Soil 1	Soil 2	Soil 3	Soil 4	Soil 5	Soil 6	Soil 7	Soil 8	Soil 9	nd	Soil 11	Soil 12
Norfloxacin	nd	16.5 (0.3)	nd	9.7 (0.2)	nd	nd	nd	nd	nd	3.6 (0.1)	nd	nd
Ciprofloxacin	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Ofloxacin	nd	nd	<MQL	nd	<MQL	<MQL	<MQL	1.8 (0.2)	<MQL	1.31 (0.1)	<MQL	<MQL
Tetracycline	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	6.9 (1.1)
Oxytetracycline	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

nd: not detected
The analyses were performed in duplicate. The standard deviation appears in parentheses.

extract that causes the suppression or enhancement of the signal in the mass spectrometer. Several strategies can be involved in such complex matrices, in order to compensate these so-called matrix effects. Each of the strategies presents advantages, but also disadvantages. Isotopic-labelled internal standards are often used to compensate the matrix effects. However, it can be difficult to find such substances affordable in the case of tetracyclines. Moreover, because of the difference between the MRM transitions of the target compounds and their respective internal standard, the compensation may not be correct [34]. The matrix-matched calibration can be used if a matrix similar to the matrix to be analyzed and free of the target compounds is available. Finally, the standard addition method is probably the most rigorous since it fits all samples and their different compositions but is also the most time-consuming approach. In the absence of samples free of the contaminants of interest (or blank samples), sludge samples were quantified by addition standards. In case the contents in the sludge exceeded the calibration range, the extracts were diluted accordingly. The soil used in the development and validation of the method was also used to build the calibration by matrix matching.

3.4. Application to Real Samples. The developed method was applied to 12 sludge samples from various sewage treatment plants in France. The results (Table 4) indicated that fluoroquinolone antibiotics were present in all the samples except one, with concentrations up to 8,492 ng/g (dry weight). The substance most frequently found and with the highest levels was ofloxacin, whose average concentration was about 1,400 ng/g. Tetracycline was also quantified in 3 sludge samples with levels comprised between 31 and 135 ng/g.

The sludge from these stations is regularly applied to agricultural soils. The soils that had been amended with these sewage sludge samples were also analyzed. Samples were collected about two months after sludge spreading. The results (Table 4) indicate the presence of fluoroquinolones at low levels in the soil, in all samples except one. Ofloxacin was present in most samples, but its concentration rarely exceeded the limit of quantification (maximum value: 1.8 ng/g). The frequency of detection of norfloxacin was lower than that of ofloxacin, but the levels were slightly higher (between 3.6 and 16.5 ng/g). These results suggest transfer of ofloxacin and norfloxacin from sludge to soil. Tetracycline was present in a single sample. Ciprofloxacin, which was quantified in most sludge samples, was not detected in soils. It should be noted, however, that the MDL of ciprofloxacin was slightly higher than the MDL of ofloxacin and norfloxacin. The absence of detectable ciprofloxacin did not exclude its presence at very low level in soil.

4. Conclusions

A method for the determination of tetracyclines and fluoroquinolones was developed, adapted to both soil and sludge matrices. This method involved extraction by PLE followed by SPE clean-up. The analysis of sludge and soil that received spreading from this sludge indicated relatively high levels of the target compounds in sludge (sometimes exceeding 1000 ng/g in the case of fluoroquinolones) and suggested transfer of certain substances between sludge and soil. The method would therefore be useful to get more information regarding this phenomenon, which is critical to assess the risks associated with the presence of tetracyclines and fluoroquinolones in biosolids.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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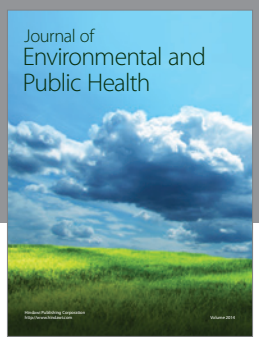

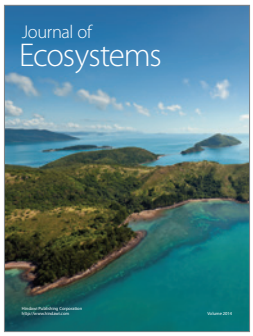

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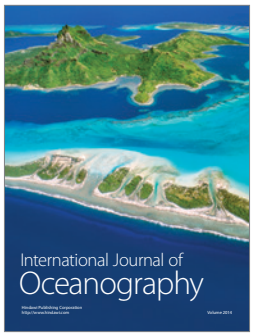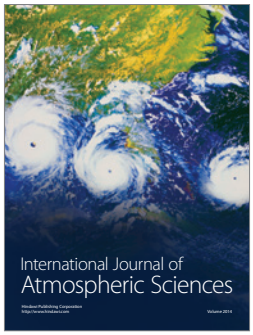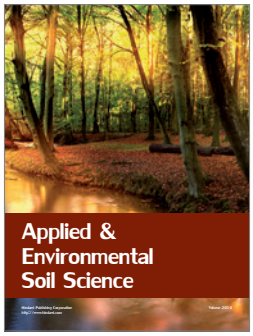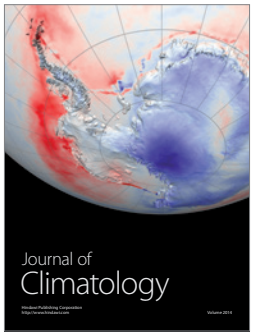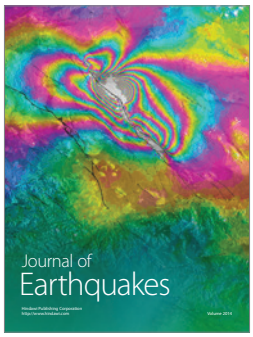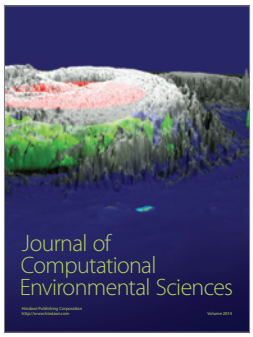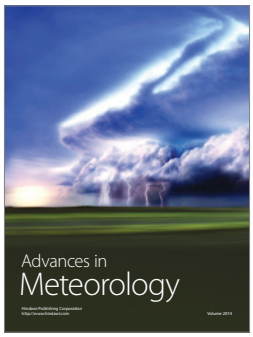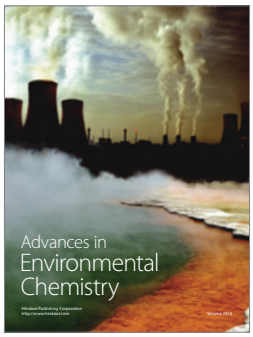
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